A dual function of SnRK2 kinases in the regulation of SnRK1 and plant growth

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Adverse environmental conditions trigger responses in plants that promote stress tolerance and survival at the expense of growth[1]. However, little is known of how stress signaling pathways interact with each other and with growth regulatory components to balance growth and stress responses. Here, we show that plant growth is largely regulated by the interplay between the evolutionarily conserved energy-sensing AMPK/SnRK1 protein kinase and the ABA (abscisic acid) phytohormone pathway. While SnRK2 kinases are major drivers of ABA-triggered stress responses, we uncover an unexpected growth-promoting function of these kinases in the absence of ABA as repressors of SnRK1. Sequestration of SnRK1 by SnRK2-containing complexes inhibits SnRK1 signaling, thereby allowing TOR activity and growth under optimal conditions. On the other hand, these complexes are essential for releasing and activating SnRK1 in response to ABA, leading to the inhibition of TOR and growth under stress. This dual regulation of SnRK1 by SnRK2 kinases couples growth control with environmental factors typical for the terrestrial habitat and is likely to have been critical for the water-to-land transition of plants.

To cope with adverse environmental conditions, plants trigger cellular and whole-plant responses that confer protection but are often detrimental to growth[1]. Despite the negative impact of stress on crop productivity, how growth is modified by stress signalling pathways is poorly understood. One major component of the stress response is SNF1-related protein kinase 1 (SnRK1), the plant ortholog of yeast SNF1 (Sucrose non-fermenting 1) and mammalian AMPK (AMP-activated protein Kinase), which drives vast metabolic and transcriptional readjustments that restore homeostasis and promote survival[2-4]. Similarly to SNF1 and AMPK, SnRK1 signaling is activated when energy levels decline during stress[2], but is also induced by abscisic acid (ABA)[5], a phytohormone essential for responses to stresses like drought, extreme temperatures or salinity[6]. In the absence of ABA, type 2C phosphatases (PP2Cs) repress subgroup III SnRK2 kinases (SnRK2.2, SnRK2.3, and SnRK2.6 in *Arabidopsis thaliana*), keeping the pathway inactive[7-11]. Binding of ABA to its receptors enables PP2C sequestration and the release and activation of SnRK2s, which thereby induce protective responses and inhibit growth[12,13].

Numerous studies have suggested cooperation between SnRK1 and ABA signaling in plant stress responses, growth and development[5,14-22], but little is known of the underlying mechanisms. SnRK1 is a heterotrimeric complex and in Arabidopsis the α-catalytic subunit is encoded by two genes, *SnRK1α1* and *SnRK1α2*. To investigate the molecular connection

between SnRK1 and ABA signaling and, given the lethality of the double *snrk1α1 snrk1α2* knockout[2,23], we generated partial *snrk1α1*$^{-/-}$ *snrk1α2*$^{+/-}$ loss-of-function mutants. These mutants show compromised SnRK1 accumulation (Supplementary Fig. 1) and signaling (Supplementary Fig. 2), as demonstrated by defective induction of SnRK1 marker genes in response to a transient dark treatment[2]. These are hereafter referred as *sesquiα2-1* or *sesquiα2-2* mutants, depending on the *snrk1α2* allele they harbor.

Despite being mostly similar to the wild-type during early development under normal conditions, *sesquiα2* mutants fail to impose an ABA-dependent post-germination growth arrest[24], developing green cotyledons in the presence of the hormone (Fig. 1a, Supplementary Fig. 3). Furthermore, *sesquiα2* mutants are unable to reduce lateral root (LR) number in response to ABA to the same extent as control plants (10%, 55%, and 41% of the mock for WT, *sesquiα2-1,* and *sesquiα2-2* seedlings, respectively; Fig. 1b). In similar assays, single *snrk1α1* and *snrk1α2* mutants are mostly indistinguishable from the wild-type, with only the *snrk1α1* mutant being mildly defective in the repression of LR growth in response to ABA (Supplementary Fig. 4). Other ABA-regulated processes, such as germination (Supplementary Fig. 5a), primary root (PR) growth (Fig. 1b), transpiration rates (Supplementary Fig. 5b), and ABA marker gene induction (Supplementary Fig. 5c) appeared normal in *sesquiα2* mutants, suggesting that the lack of SnRK1 affects only specific ABA responses and/or that SnRK1 signaling is not sufficiently compromised to visibly affect all ABA-related processes. Importantly, *sesquiα2* mutants fail to repress LR growth also under low light conditions (Supplementary Fig. 6), showing that defective growth inhibition is not exclusive to ABA, and that, given the weak nature of this mutant, its defects are only apparent under conditions that substantially compromise growth in WT plants.

Given that all the observed ABA phenotypes of the SnRK1 *sesquiα2* mutants relate to growth repression, and given the known antagonistic relationship between AMPK/SnRK1 and the growth-promoting Target of Rapamycin (TOR) kinase in animals[25] and possibly in plants[4], we examined the activation status of TOR in the *sesquiα2-1* mutant in response to ABA. The phosphorylation of ribosomal protein S6 (RPS6^{S240}) in whole seedling extracts served as a faithful readout[26], confirming previous results on the inhibition of TOR signaling by ABA and its dependency on SnRK2 kinases[27] (Supplementary Fig. 7). In response to ABA, the *sesquiα2-1* mutant showed a slower inhibition of TOR along all the analyzed 4h time-course sampling points (Fig. 1c), indicating that SnRK1α1 is required for repressing TOR activity in response to ABA. To assess if the SnRK1α effect is direct, we next analyzed

the physical interaction between SnRK1α1 and TOR by co-immunoprecipitation (co-IP), using a GFP-tagged SnRK1α1 line[14], a *35S::GFP* control line, and antibodies recognizing TOR or its regulatory protein RAPTOR. In whole seedling extracts TOR was readily co-immunoprecipitated with SnRK1α1-GFP (Fig. 1d) but not with GFP alone (Fig. 1e). A basal SnRK1α1-TOR interaction was detected in mock conditions, and it was enhanced two-fold by a short ABA treatment (40 min; Fig. 1d). Similar results were obtained for RAPTOR (Supplementary Fig. 8a-b), confirming previous observations that SnRK1α1 and RAPTOR interact *in planta*[4,28]. These results were further corroborated for the endogenous proteins using TOR immunoprecipitation and immunodetection of SnRK1α1 (Supplementary Fig. 8d). A recent study demonstrated that the repression of TOR by ABA is SnRK2-dependent[27]. However, using a GFP-tagged SnRK2.2 line[29] we were unable to detect any interaction of TOR or RAPTOR with SnRK2.2-GFP either in mock- or ABA-treated plants (Fig. 1f and Supplementary Fig. 8c). Furthermore, none of the three SnRK2s (SnRK2.2/2.3/2.6) could be detected in immunoprecipitates of endogenous TOR in either of the two conditions (Supplementary Fig. 8d), altogether suggesting that, despite being necessary for repressing TOR in response to ABA[27], SnRK2s may not be directly involved in TOR repression and that TOR is instead inhibited by SnRK1.

To explore the molecular connection between SnRK2 and SnRK1, we first examined their potential co-localization. As previously reported, SnRK1α1 and SnRK2.2 were prominently expressed in the root tip, in LR primordia and in subsequent stages of LR development (Supplementary Fig. 9)[14,29]. At the subcellular level both kinases were present in the cytosol and the nucleus, being particularly enriched in the latter (Supplementary Fig. 9). To investigate the SnRK1-SnRK2 physical interaction we next performed reciprocal co-IP experiments using the same material and conditions as for the microscopy analyses (roots, 3h ABA treatment). In mock-treated seedlings we retrieved a clear interaction between SnRK1α1 and SnRK2 in both directions (Fig. 2a-2b), whilst neither SnRK2 nor SnRK1α1 could be detected in immunoprecipitates of GFP alone (Supplementary Fig. 10a). The reported interaction of both SnRK2[9,10] and SnRK1α1[5] with clade A PP2C phosphatases served as positive controls (Fig. 2c-d). Strikingly, treatment with ABA caused a marked reduction in all three interactions (Fig. 2a-d; for the PP2CA interactions please note that this is relative to the total PP2CA amount, which is known to be strongly increased by ABA through transcriptional activation[30]), suggesting that the three proteins may be part of the same complexes. A similar effect of ABA on the SnRK2-SnRK1α1 interaction was observed using the same material and conditions as for evaluating the interaction with TOR (whole seedlings,

40 min ABA treatment; Supplementary Fig. 10b-c), showing the interaction is rapidly reduced by the hormone. Using seedlings overexpressing FLAG-tagged SnRK2.3 and SnRK2.6 we could further demonstrate that the interaction between SnRK1α1 and SnRK2s as well as the reduction of this interaction by ABA is shared by all three ABA-induced SnRK2 kinases (Supplementary Fig. 10d-e).

To assess whether the interaction between SnRK1 and SnRK2 is direct or whether it is dependent on the presence of PP2Cs we used bimolecular fluorescence complementation (BiFC) assays in *Nicotiana benthamiana* (Fig. 2e and Supplementary Fig. 11a-b). Expression of YFP^N-SnRK1α1 with YFP^C-SnRK2s and a nuclear targeted RFP control (mRFP-NLS) did not result in YFP reconstitution (Fig. 2e and Supplementary Fig. 11a-b). However, co-expression of the two kinases with PP2CA-RFP yielded a very strong YFP signal in the nucleus, indicating that the presence of PP2CA enables SnRK2s to interact with SnRK1α1. Moreover, a kinase dead SnRK2.6 variant [$SnRK2.6^{G33R}$][31] was also able to interact with SnRK1α1 in a PP2CA-dependent manner, demonstrating that the SnRK1α1-SnRK2 interaction does not rely on the kinase activity of the latter (Supplementary Fig. 11a-b). Immunoblot analyses of the infiltrated leaf sectors confirmed the expression of YFP^N-SnRK1α1 and YFP^C-SnRK2s in all samples (Supplementary Fig. 11c).

To investigate the relationship between SnRK1 and SnRK2 kinases we crossed the *snrk1α1* single mutant to the *snrk2.2/2.3* double mutant (hereafter referred as *snrk2d*) to assess their genetic interaction (Supplementary Fig. 12). We reasoned that, given the partial impairment of ABA responses in this mutant[7] [as opposed to the full impairment of the *snrk2.2/2.3/2.6* mutant (*snrk2t*)[32-34]], a potential contribution from the *snrk1α1* mutation could be more easily detected in this background. Despite having mostly no effect on its own (Supplementary Fig. 4), the *snrk1α1* mutation clearly enhanced the ABA insensitivity of the *snrk2d* mutant, increasing its germination and cotyledon greening rates (Fig. 3a-b), and the formation of LRs in ABA (Fig. 3c). This indicates that the SnRK1 pathway contributes to specific ABA signaling outputs. Furthermore, the sensitization of the *snrk1α1* mutation by the *snrk2d* background in ABA, suggests that SnRK2s may promote SnRK1 signaling in these conditions. To investigate whether SnRK2s can phosphorylate and activate SnRK1 directly, we first immunoprecipitated active and inactive HA-tagged SnRK2.3 variants expressed in Arabidopsis mesophyll protoplasts treated under mock or ABA conditions. Selective activation of SnRK2.3 by ABA was validated using a *RD29B::LUC* reporter assay[35] (Supplementary Fig. 13a). Immunoprecipitated proteins were tested in an *in vitro* SnRK1α1 kinase assay using a similarly generated SnRK1 upstream kinase (SnAK2[36]). Whilst

incubation of recombinant SnRK1α1 with immunoprecipitated SnAK2 resulted in a strong induction of SnRK1 activity, no effect was observed for the ABA-activated SnRK2.3, which yielded similarly low SnRK1 activities as the inactive SnRK2.3^{K51N} variant (Supplementary Fig. 13b-c). Altogether, these results suggest that SnRK2s promote SnRK1 signaling but this does not appear to involve direct SnRK1α1 activation.

We next asked whether repression of TOR by SnRK1 always requires SnRK2s or whether this requirement is specific to ABA. To address this, we compared the inhibition of TOR by a dark-induced energy deficit in control plants, *sesquiα2-1*, and *snrk2t* mutants. As expected, *sesquiα2-1* seedlings had a reduced capacity to repress RPS6^{S240} phosphorylation in response to darkness (Supplementary Fig. 14a). This is consistent with previous reports showing defective repression of TOR outputs in plants that have compromised SnRK1 signaling[4]. However, the *snrk2t* mutant displayed similar kinetics in the repression of TOR signaling as the wild-type (Supplementary Fig. 14b), supporting the idea that SnRK2s are only required for repressing TOR *via* SnRK1 in response to ABA but not energy depletion.

We noticed that, despite its ABA insensitivity and overall increased growth in ABA, the *snrk2d* mutant displayed reduced PR and LR growth in control plates compared to the WT (Fig. 3c), in accordance with a previous report[29]. Most strikingly, this was fully rescued by the *snrk1α1* mutation, indicating that the reduced growth of the *snrk2d* mutant is SnRK1α1-dependent and suggesting that, in the absence of ABA, SnRK2s promote root growth by repressing SnRK1α1 (Fig. 3c). Further supporting a growth-promoting function of SnRK2s in normal conditions, a line overexpressing SnRK2.3 had longer PR in control plates (Supplementary Fig. 15), whilst showing enhanced repression of PR growth in ABA, in accordance with its known ABA hypersensitivity[37]. To assess whether the differences in growth observed in mock conditions are TOR-dependent, we grew seedlings in increasing concentrations of the TOR inhibitor AZD8055. The *snrk2d* mutant displayed a clear hyposensitivity to AZD, with differences in PR length between WT and *snrk2d* seedlings being strongly reduced under increasing concentrations of the inhibitor (Fig. 3d). Furthermore, a normal sensitivity to AZD was restored by the *snrk1α1* mutation, indicating that the lower TOR activity of the *snrk2d* mutant is SnRK1-dependent (Fig. 3d). To further explore how the interplay between SnRK2 and SnRK1 kinases affects TOR activity, we performed a time-course experiment to monitor the induction of RPS6 phosphorylation in response to nutrient supplementation (replacement of the growth medium with fresh medium; Fig. 3e). In WT seedlings a marked increase in RPS6 phosphorylation was detected within the first 30 min of refreshing the medium, followed by a slight decrease and stabilization after 1h.

In the *snrk2d* mutant, however, the induction of RPS6 phosphorylation was defective, but this defect was fully rescued by the *snrk1α1* mutation. Altogether this and the AZD sensitivity experiment show that in the *snrk2d* mutant TOR is repressed to a higher extent than in WT plants and that this overrepression is SnRK1-dependent. These results further suggest that in the absence of SnRK2s, basal SnRK1 activity is increased. To investigate this, we analyzed WT and *snrk2d* seedlings with regard to the phosphorylation status of TREHALOSE PHOSPHATE SYNTHASE 5 (TPS5), a established direct target of SnRK1[38,39]. The *tps5-1* mutant is a knockout for TPS5[40] and served as a control for the specificity of the TPS5 antibody (Fig. 3f). We found that the levels of TPS5 phosphorylation were indeed higher in the *snrk2d* mutant (1.7-fold), consistent with an enhanced SnRK1 activity. To explore this further we immunoprecipitated SnRK1α1 from WT and *snrk2d* seedlings and analyzed its interaction with the SnRK1β1 regulatory subunit. The β-regulatory subunits are considered to act as scaffolds in the SnRK1 complex, being crucial for the recruitment of specific targets[41]. The SnRK1β1 subunit, in particular, has been implicated in the control of nitrogen and carbon metabolism[42] and we therefore reasoned it could be involved in the regulation of TOR and TPS5 by the SnRK1 complex. The interaction of SnRK1α1 with the SnRK1β1 subunit was indeed higher (1.7-fold) in the *snrk2d* mutant (Fig. 3g), suggesting that the lower TOR activity and increased TPS5 phosphorylation of this mutant could be the result of enhanced engagement of the SnRK1β1 subunit.

We conclude that SnRK2 kinases perform dual functions in plants (Fig. 4). In the absence of ABA, SnRK2s promote growth: SnRK2s are required, together with PP2Cs, to form "repressor complexes" that sequester SnRK1, precluding its interaction with TOR and thereby the inhibition of TOR signaling and growth. Sequestration of SnRK1α1 in these complexes is important for root growth (in the case of SnRK2.2 and SnRK2.3), and may potentially explain other reported unexpected effects of SnRK2 kinases, including SnRK2.6, in promoting metabolism, growth, and development in optimal conditions[43,44]. We propose that these complexes are the same as the ones performing canonical ABA signaling functions and that their disassembly requires sequestration of the PP2C repressors by the ABA-bound ABA receptors. Several lines of evidence support this. First, likewise SnRK2s[45], the activation of SnRK1 by ABA requires relief of inhibition by PP2C phosphatases[5]. Second, ABA reduces the interaction of SnRK1α1 with SnRK2 and PP2CA and between SnRK2 and PP2CA (Figs 2a-d, Supplementary Fig. 10b-c). Third, SnRK1α1 and SnRK2 are unable to interact in the absence of PP2Cs (Fig. 2e). Forth, SnRK2s (SnRK2.2/SnRK2.3/SnRK2.6) are

absolutely required for repressing TOR in response to ABA[27] (Supplementary Fig. 7b), even though SnRK2s may be involved in TOR repression only indirectly.

In the presence of ABA, SnRK2s repress growth and this is partly accomplished by enabling SnRK1 activation by the hormone (Fig. 4): SnRK1 repressor complexes harboring SnRK2s and PP2Cs dissociate through canonical ABA signaling, releasing SnRK1α1 and SnRK2 to activate stress responses. One major consequence of the ABA-triggered disassembly of these complexes is the interaction of released SnRK1α1 with TOR, ultimately leading to growth inhibition. In the absence of SnRK2s these repressor complexes are not formed, rendering SnRK1 and the repression of TOR insensitive to ABA. In agreement with this, Arabidopsis *raptor and lst8* mutants are ABA hypersensitive with regard to germination, early seedling development, and root growth[46,47] whilst TOR overexpressors in rice display ABA insensitivity during germination[48]. The fact that the ABA sensitivity of the *sesquiα2* mutants was only manifested at the level of cotyledon greening and LR density but not at the level of germination or PR length (Fig. 1), is likely to be explained by the weak nature of these mutants (Supplementary Fig. 2), by the fact that germination had to be scored from a segregating seed population and by the fact that LRs are more sensitive to ABA than the PR[49]. Repression of TOR in response to ABA may also require active input from SnRK2[27]. However, given the lack of interaction between SnRK2s and TOR *in planta* (Fig. 1f and Supplementary Fig. 8), the simple requirement of SnRK2s to form SnRK1 repressor complexes that disassemble in response to ABA may be sufficient to explain why SnRK2s are essential for growth repression by this hormone[27].

Repression of SnRK1 by SnRK2 and PP2C allows SnRK1 to be released and activated in response to ABA. However, SnRK1 is also regulated by energy depletion through mechanisms that are SnRK2-independent (Supplementary Fig. 14), suggesting that SnRK1 associates with different factors that enable its activation in response to specific signals. We propose that, in addition to its ancient and highly conserved energy-sensing function, SnRK1 evolved in land plants to respond to ABA, a crucial signal for survival in terrestrial habitats. Intriguingly, this is accomplished through repression by the phylogenetically related subgroup III SnRK2 kinases, which belong to the same SnRK superfamily as SnRK1[50], but are specific to land plants[51,52]. Coupling the ABA-PP2C-SnRK2 module to the evolutionarily conserved SnRK1-TOR axis conferred plants the capacity to regulate growth in response to water availability and may have represented a steppingstone for the establishment of terrestrial life.

MATERIALS AND METHODS

A list of all primers, antibodies, and plant lines used in this study is provided in Table S1.

Plant material and growth

All *Arabidopsis thaliana* plants used in this study are in the Columbia (Col-0) background. Unless otherwise specified, plants were grown under long-day conditions (16h light, 100 µmol $m^{-2}s^{-1}$, 22°C /8h dark, 18°C) on 0.5X MS medium (0.05% MES and 0.8% phytoagar). The *sesquiα2-1* (*snrk1α1-3*$^{-/-}$ *snrk1α2-1*$^{+/-}$) and *sesquiα2-2* (*snrk1α1-3*$^{-/-}$ *snrk1α2-2*$^{+/-}$) mutants were obtained by crossing the *snrk1α1-3* (GABI_579E09) with the *snrk1α2-1* (WiscDsLox320B03) and *snrk1α2-2* (WiscDsLox384F5) mutants, respectively. *sesquiα2* individuals were always pre-selected on BASTA-containing medium for 5-6 days together with a BASTA-resistant *35S::GFP* line [referred as Col(B) in the text], except for germination and early development assays. Triple *snrk2.2/snrk2.3/snrk1α1-3* mutants (referred as *snrk2d*/*α1* in the text) were obtained by crossing *snrk1α1-3* to the *snrk2.2/snrk2.3* double mutant (*snrk2d*) [7].

Phenotype Assays

For assays of ABA sensitivity during germination and early seedling development, seeds were plated on 0.5X MS supplemented or not with ABA, and radicle emergence and cotyledon greening were computed over time under a stereoscope.

For assaying ABA sensitivity during root development, seedlings were grown vertically for 6 days in 0.5X MS (supplied with BASTA in experiments with the *sesquiα2* mutant) and transferred to 0.5X MS plates supplemented or not with ABA for 8 more days. All computed parameters relate to the region of the root that developed after transferring the seedlings to new mock or ABA plates. For LRs only those ≥ 0.5 mm long were considered.

Co-immunoprecipitation experiments

Interaction of SnRKs with TOR and RAPTOR

For assessing the interaction of SnRKs with TOR and RAPTOR, seedlings (*proSnRK1α1::SnRK1α1-GFP*, *proSnRK2.2::SnRK2.2-GFP* and *35S::GFP*) were grown on 0.5X MS + 0.5% sucrose for 14d (7d in solid medium and 7d in liquid culture) and treated with 50 µM ABA for 40 min. GFP-tagged proteins were immunoprecipitated from whole seedling cleared protein extracts using super-paramagnetic µMAC beads coupled to monoclonal anti-GFP antibody (Miltenyi Biotec), and co-immunoprecipitated proteins were

analyzed by Western blotting using anti-GFP, anti-TOR, anti-RAPTOR, anti-SnRK1α1 and anti-SnRK2 antibodies.

For immunoprecipitation of endogenous TOR, the anti-TOR antibody was coupled to Dynabeads™ Protein A (Invitrogen™) prior to its addition to the whole seedling cleared protein extracts. Co-immunoprecipitated proteins were analyzed by Western blot with anti-TOR, anti-SnRK1α1 and anti-SnRK2s antibodies.

Interaction of SnRK1 with SnRK2 and PP2CA

For assessing the interaction of SnRK1 with SnRK2 and PP2CA, seedlings (*proSnRK1α1::SnRK1α1-GFP*, *proSnRK2.2::SnRK2.2-GFP* and *35S::GFP*) were grown on 0.5X MS + 0.5% sucrose for 14d (7d in solid medium and 7d in liquid culture), and roots were rapidly harvested following a 3h treatment with 50 μM ABA. GFP-tagged proteins were immunoprecipitated from cleared protein extracts using super-paramagnetic μMAC beads coupled to monoclonal anti-GFP antibody (Miltenyi Biotec), and co-immunoprecipitated proteins were analyzed by Western blotting using anti-GFP, anti-SnRK1α1, anti-SnRK2, and anti-PP2CA[30] antibodies. When indicated, the SnRK1-SnRK2 interaction was analyzed also from whole seedlings following a 40 min treatment with 50 μM ABA as explained above for the interaction with TOR.

RPS6^{S240} phosphorylation assays

Seedlings were grown on 0.5X MS + 0.5% sucrose for 12 d (6 d in solid medium ± BASTA and 6d in liquid culture) and treated with mock, 50 μM ABA, 10 μM torin2 or 2 μM AZD8055 during 4 h. For the ABA time course, ABA (50 μM) was added 1 h after the onset of the lights and samples were collected immediately (T0) or after 15, 30, 45, 60 and 240 min. For the nutrient supplementation time course, the growth medium (0.5X MS + 0.5% sucrose) was replaced with fresh medium 1 h after the onset of the lights and seedlings were immediately collected (T0) or after 30, 60 and 180 min. For the sudden darkness experiments, samples were collected 3h after the onset of the lights (T0) or after 1 or 3 h of incubation in the dark. Samples were analyzed by Western Blot with anti-phospho-RPS6^{S240} and anti-RPS6 antibodies.

Custom-made SnRK1α1 and SnRK1α2 antibodies

Polyclonal *Arabidopsis* SnRK1α1 and SnRK1α2 antibodies were obtained by conjugating synthetic peptides (CTMEGTPRMHPAESVA and CTTDSGSNPMRTPEAGA, respectively;

produced by Cocalico Biologicals, Inc. USA) to keyhole limpet hemocyanin and injecting two rabbits (performed by Cocalico Biologicals). Antibodies were affinity-purified using the original peptides linked to a SulfoLink matrix (Pierce) following instructions by the manufacturer.

Data availability

All data supporting the findings of this study are available in the main text or the Supplementary Information. Additional data related to this study are available from the corresponding author upon request. All biological materials used in this study are available from the corresponding author on reasonable request.

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FIGURE LEGENDS

Fig. 1. SnRK1 *sesquiα2* mutants show defective growth repression in ABA. a, SnRK1 *sesquiα2-1* and *sesquiα2-2* mutants have higher cotyledon greening rates than control plants in ABA. Graph shows the percentage of green and expanded cotyledons in seedlings grown for 15d on 0.5X MS with or without ABA (n=3, 100 seeds per genotype each experiment; error bars, SEM). *p*-values denote statistically significant differences for comparisons to the Col-0 control (one-way ANOVA with Tukey HSD test). **b,** SnRK1 *sesquiα2-1* and *sesquiα2-2* mutants have higher lateral root (LR) density than control plants in ABA. Left panels, representative pictures of seedlings grown vertically on 0.5X MS medium with BASTA for 5d and transferred to 0.5X MS with or without ABA for 8d. Right panels, quantification of primary root (PR) length and LR density from 6 independent experiments (total number of plates: WT mock n=16, *sesquiα2-1* mock n=7, *sesquiα2-2* mock n=9, WT ABA n=24, *sesquiα2-1* ABA n=12, *sesquiα2-2* mock n=12; total number of seedlings: 36-72 per genotype and condition). Upper and lower box boundaries represent the first and third quantiles, respectively, horizontal lines mark the median and whiskers mark the highest and lowest values. *p*-values denote statistically significant differences for comparisons to control plants (one-way ANOVA with Tukey HSD test). Col(B), BASTA-resistant Col-0 expressing *35S::GFP*, used as control. **c,** Repression of TOR signaling in response to ABA is slower in SnRK1 *sesquiα2-1* mutants than in Col(B) control plants. Seedlings were treated with 50 μM ABA for the indicated times and TOR activity was subsequently analyzed from total protein extracts using immunoblotting and $RPS6^{S240}$ phosphorylation as readout. Graph corresponds to the average of 5 independent experiments (error bars, SEM). *p*-values denote statistically significant differences (two-tailed Welch t-test). All samples were run in the same gel but images were cropped for showing first the Col(B) series. **d,** TOR interacts with SnRK1α1 and the interaction is enhanced two-fold in ABA. 14d-old seedlings expressing SnRK1α1-GFP, were treated with mock or 50 μM ABA for 40 min, GFP-tagged proteins were immunoprecipitated from total protein extracts and co-immunoprecipitation of TOR was assessed by immunodetection with TOR specific antibodies. Two independent experiments are shown. Numbers refer to the relative intensity of the corresponding TOR band. **e, f,** TOR is not co-immunoprecipitated with GFP alone (**e**) or with SnRK2.2-GFP (**f**). 14d-old seedlings expressing *35S::GFP* or *proSnRK2.2::SnRK2.2-GFP* were treated and analyzed as in (**d**). Two independent experiments were performed with similar results (**e**, **f**).

Fig. 2. SnRK2s interact with SnRK1 in a PP2CA-dependent manner. a, b, SnRK1α1 and SnRK2.2 interact *in planta* and the interaction is reduced over 2-fold in ABA. Seedlings expressing *proSnRK1α1:SnRK1α1-GFP* (**a**) or *proSnRK2.2:SnRK2.2-GFP* (**b**) were mock- or ABA-treated, GFP-tagged proteins were immunoprecipitated from roots and co-immunoprecipitation of SnRK2 and SnRK1α1, respectively was assessed by immunodetection with the indicated antibodies. Graphs correspond to the average of 4 independent experiments (error bars, SEM). *p*-values denote statistically significant differences (**a**, two-tailed Student t-test, **b**, two-tailed Welch t-test). **c, d,** PP2CA co-immunoprecipitates with SnRK1α1-GFP (**c**) and SnRK2.2-GFP (**d**) and, proportionally to the total PP2CA levels, both interactions are reduced in ABA. Seedlings expressing *proSnRK1α1::SnRK1α1-GFP* or *proSnRK2.2::SnRK2.2-GFP* were mock- or ABA-treated, GFP-tagged proteins were immunoprecipitated from roots and co-purifying proteins were analyzed by immunoblotting with specific antibodies. Two independent experiments were performed with similar results (**c**, **d**). **e,** BiFC experiments show that SnRK1α1 and SnRK2.2 interact only in the presence of PP2CA and this interaction occurs mostly in the nucleus. Left panels, representative pictures of *Nicotiana benthamiana* epidermal cells expressing YFP^N-SnRK1α1 and YFP^C-SnRK2.2 with a nuclear localized RFP (mRFP-NLS) or with PP2CA-RFP. Right panels, quantification of RFP and YFP signals (error bars, SEM; mRFP-NLS samples, n=9; PP2CA-RFP samples, n=14). Scale bars, 30 μm. Two independent experiments were performed with similar results.

Fig. 3. SnRK2s regulate TOR and growth *via* SnRK1. a, The *snrk1α1-3* mutation increases the ABA insensitivity of the *snrk2d* mutant during germination. Upper two panels, seeds of Col-0, *snrk2d*, and *snrk2d snrk1α1* (*snrk2d/1α1*) mutants were plated on 0.5X MS with or without ABA and radicle emergence was scored at the indicated times (shown are percentages in ABA as compared to the mock condition; n=3, 50 seeds per genotype each experiment; error bars, SEM). Different letters indicate statistically significant differences for each time point ($p<0.05$, one-way ANOVA with Tukey HSD test). Lower panel, degree of ABA insensitivity computed by normalizing the parameters scored in ABA to the corresponding mock control (error bars, SEM). *p*-values refer to the differences between *snrk2d/1α1* and *snrk2d* (one-way ANOVA with Tukey HSD test for each time point). **b,** The *snrk1α1-3* mutation increases the cotyledon greening rates of the *snrk2d* mutant in ABA. Seeds were plated as in (**a**) and cotyledon greening was scored after 16d. Graph corresponds to the

536 average of 3 independent experiments (100 seeds per genotype each experiment; error bars,
537 SEM). *p*-values denote statistically significant differences (two-tailed Student t-test). **c,** In
538 control conditions the *snrk2d* mutant has defects in primary (PR) and lateral root (LR) growth
539 that are fully rescued by the *snrk1α1* mutation. In ABA the *snrk1α1* mutation enhances the
540 ABA hyposensitivity of the *snrk2d* mutant with regard to PR length and LR density. Upper
541 panel, representative picture of seedlings grown vertically on 0.5X MS medium for 5d and
542 transferred to 0.5X MS with or without ABA for 8d. Middle panels, quantification of PR
543 length and LR density from 3 independent experiments (total number of plates: WT mock
544 n=21, *snrk2d* mock n=19, *snrk2d/1α1* mock n=21, WT ABA n=21, *snrk2d* ABA n=21,
545 *snrk2d/1α1* ABA n=21; total number of seedlings: 37-42 seedlings per genotype and
546 condition). Upper and lower box boundaries represent the first and third quantiles,
547 respectively, horizontal lines mark the median and whiskers mark the highest and lowest
548 values. Lower panels, degree of ABA insensitivity computed by normalizing the parameters
549 scored in ABA to the corresponding mock control (error bars, SEM). Different letters indicate
550 statistically significant differences ($p<0.05$, one-way ANOVA with Tukey HSD test). **d,** The
551 *snrk2d* mutant exhibits hyposensitivity to TOR inhibition by AZD8055 and this is fully
552 rescued by the *snrk1α1* mutation. Left panel, representative pictures of seedlings grown
553 vertically on 0.5X MS medium for 7d and transferred to 0.5X MS with or without the
554 indicated AZD concentrations for 7d. Percentage values refer to the average increment in PR
555 length (from the point of transfer) of the *snrk2d* as compared to that of the WT in each
556 condition. Right panel, quantification of primary root (PR) length from 2 independent
557 experiments (total number of plates per genotype: mock, n=12; 0.2 μM AZD, n=11, 0.5 μM
558 AZD, n=10; total number of seedlings: 20-24 per genotype and condition; error bars, SEM).
559 Different letters indicate statistically significant differences ($p<0.0001$, two-way ANOVA
560 with Tukey´s HSD test). **e,** The *snrk2d* mutant shows defective induction of TOR signaling
561 and this is fully rescued by the *snrk1α1* mutation. Samples were collected at the indicated
562 times following replacement of the growth medium with fresh medium (FM). TOR activity
563 was analyzed from total protein extracts using immunoblotting and $RPS6^{S240}$ phosphorylation
564 as readout. Graph corresponds to the average of 5 independent experiments (error bars, SEM).
565 Different letters indicate statistically significant differences for each time point ($p<0.05$, one-
566 way ANOVA with Tukey HSD test). **f,** The *snrk2d* mutant shows higher phosphorylation of
567 TPS5, indicating higher SnRK1 activity. WT and *snrk2d* seedlings were grown as in panel (**c**)
568 (only mock conditions). Whole seedlings were harvested and total protein extracts were
569 analyzed using Phos-tag gels to separate TPS5 phospho-proteoforms from the non-

phosphorylated protein, followed by immunoblotting with a TPS5 antibody (lower panel). Extracts from the *tps5-1* mutant were included in regular Western blot analyses (upper pannel) as control for the specificity of the TPS5 antibody. All samples were run in the same gel but images were cropped for showing *tps5-1* alongside WT and *snrk2d*. Graph corresponds to the average of 3 independent experiments (error bars, SEM). **g,** The interaction between SnRK1α1 and the SnRK1β1 regulatory subunit is enhanced in the *snrk2d* mutant. SnRK1α1 was immunoprecipitated from total protein extracts of 14d-old WT and *snrk2d* seedlings and co-purifying proteins were analyzed by immunoblotting with a SnRK1β1 antibody. Graph corresponds to the average of 3 independent experiments (error bars, SEM). *p*-values denote statistically significant differences (**f**, two-tailed ratio t-test; **g**, two-tailed Student t-test).

Fig. 4. A dual function of SnRK2 kinases in the regulation of SnRK1 and growth. Upper panel: under optimal conditions, SnRK2s promote growth. In the absence of ABA, SnRK2s are required for the formation of SnRK1 repressor complexes that harbor also PP2Cs. Sequestration of SnRK1 in these complexes is important to prevent its interaction with TOR and thereby to allow growth when conditions are favorable. Lower panel: under stress conditions, SnRK2s inhibit growth. In the presence of ABA, SnRK2 and PP2C-containing SnRK1 repressor complexes disassemble through canonical ABA signaling involving the sequestration of PP2Cs by the ABA-bound PYR/PYL receptors. Disassembly of the complexes releases SnRK2s and SnRK1α to trigger stress responses and inhibit growth. This is partly accomplished by direct TOR repression by SnRK1 but may also involve co-participation of SnRK2 kinases. Inactive components are shown in white. Dark blue and dark orange denote components that are active under optimal conditions or under stress, respectively.

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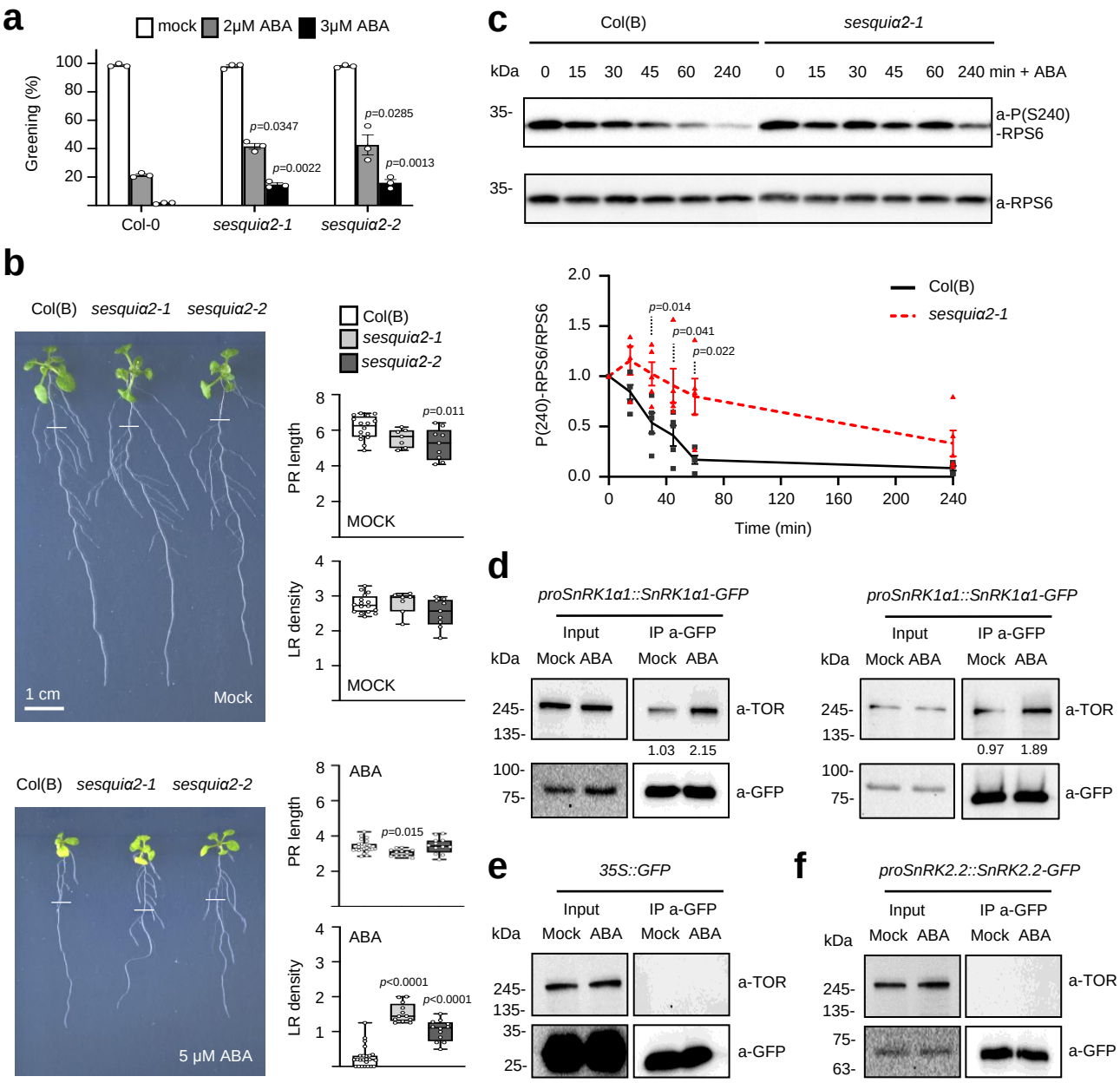
This work is dedicated to the memory of our beloved friend and colleague Américo Rodrigues.

Author contributions

BBP, MA, and CV designed and performed experiments, and analyzed and interpreted data. LF performed and analyzed the root phenotyping experiments in low light and AZD. AC generated and characterized molecularly the *sesquiα2* mutant lines and provided strong conceptual support. DRB performed protein immunoprecipitation from protoplasts and *in vitro* kinase assays. AR contributed to the general conception of the project and the initial exploratory experiments. CM contributed the phospho-RPS6 antibody and expertise on molecular and plant phenotype assays related to TOR activity. PLR contributed tools and expertise on PP2C-SnRK2 interactions and ABA signaling, and actively supported the conceptual work. BBP and EBG prepared the figures and wrote the manuscript. EBG conceived the project and directed and supervised all of the research. All authors read and approved the manuscript.

Figure 1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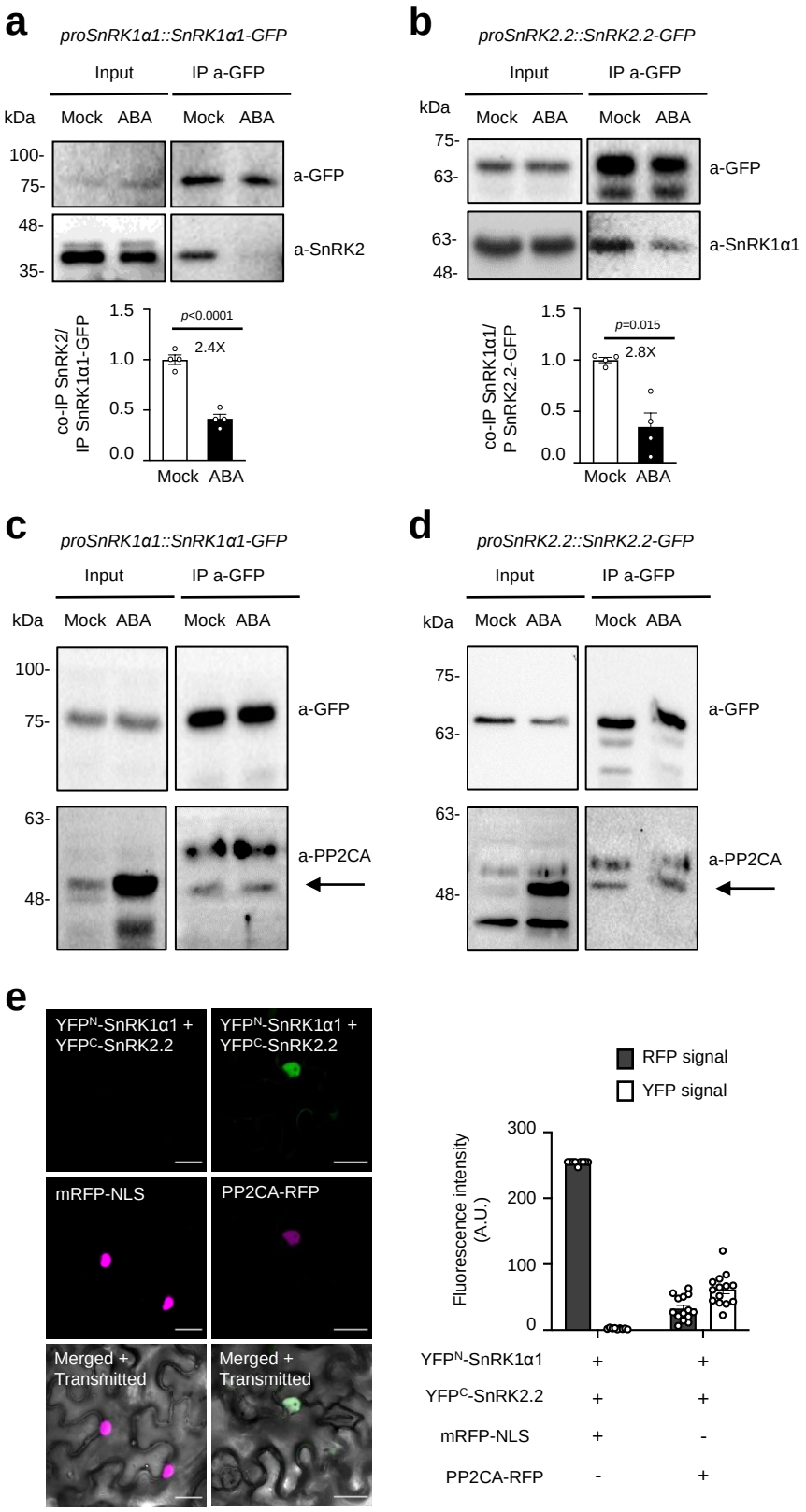
mock
2μM ABA
3μM ABA
Greening (%)
p=0.0347
p=0.0022
p=0.0285
p=0.0013
Col-0
sesquia2-1
sesquia2-2
b
Col(B)
sesquia2-1
sesquia2-2
1 cm
Mock
5 μM ABA
PR length
LR density
MOCK
ABA
p=0.011
p=0.015
p<0.0001
c
Col(B)
sesquia2-1
kDa
0 15 30 45 60 240 0 15 30 45 60 240 min + ABA
a-P(S240)-RPS6
a-RPS6
P(240)-RPS6/RPS6
Time (min)
p=0.014
p=0.041
p=0.022
d
proSnRK1α1::SnRK1α1-GFP
Input
IP a-GFP
Mock ABA Mock ABA
a-TOR
a-GFP
1.03 2.15
0.97 1.89
e
35S::GFP
f
proSnRK2.2::SnRK2.2-GFP

Figure 2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kDa
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ABA
Mock
ABA
100-
75-
48-
35-
a-GFP
a-SnRK2
co-IP SnRK2/
IP SnRK1α1-GFP
1.5
1.0
0.5
0.0
p<0.0001
2.4X
Mock
ABA
b
proSnRK2.2::SnRK2.2-GFP
Input
IP a-GFP
kDa
Mock
ABA
Mock
ABA
75-
63-
63-
48-
a-GFP
a-SnRK1α1
co-IP SnRK1α1/
P SnRK2.2-GFP
1.5
1.0
0.5
0.0
p=0.015
2.8X
Mock
ABA
c
proSnRK1α1::SnRK1α1-GFP
Input
IP a-GFP
kDa
Mock
ABA
Mock
ABA
100-
75-
63-
48-
a-GFP
a-PP2CA
d
proSnRK2.2::SnRK2.2-GFP
Input
IP a-GFP
kDa
Mock
ABA
Mock
ABA
75-
63-
63-
48-
a-GFP
a-PP2CA
e
YFPN-SnRK1α1 +
YFPC-SnRK2.2
YFPN-SnRK1α1 +
YFPC-SnRK2.2
mRFP-NLS
PP2CA-RFP
Merged +
Transmitted
Merged +
Transmitted
RFP signal
YFP signal
Fluorescence intensity
(A.U.)
300
200
100
0
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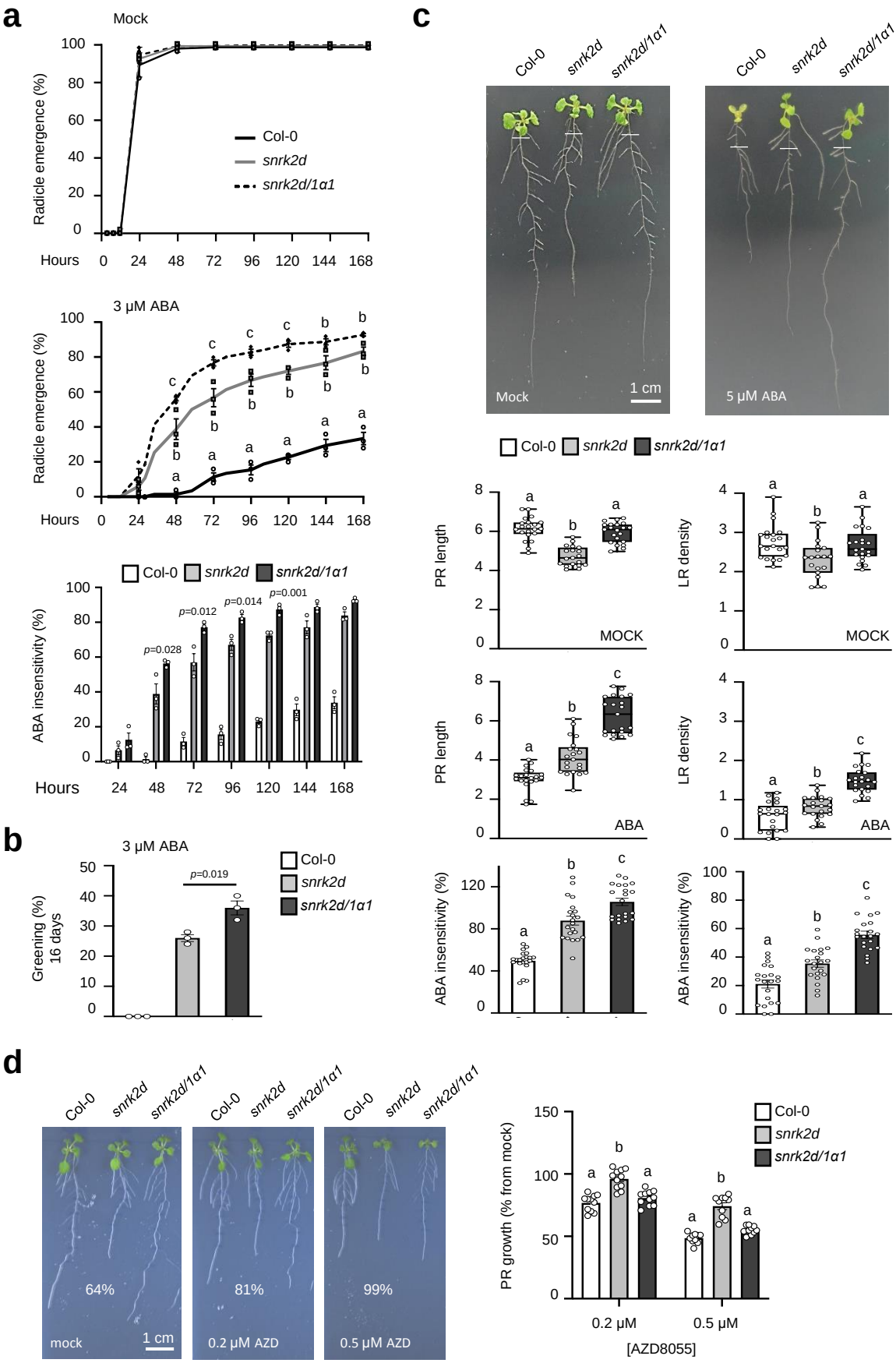
YFPC-SnRK2.2 + +
mRFP-NLS + -
PP2CA-RFP - +

Figure 3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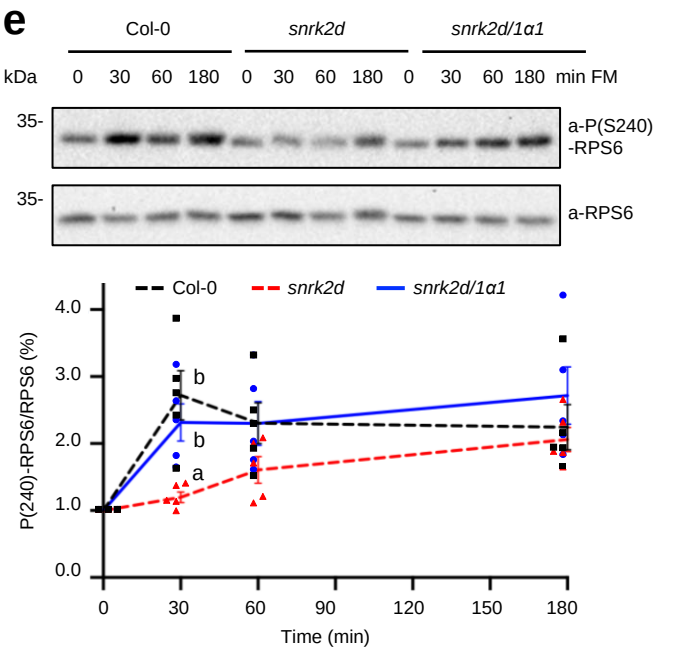
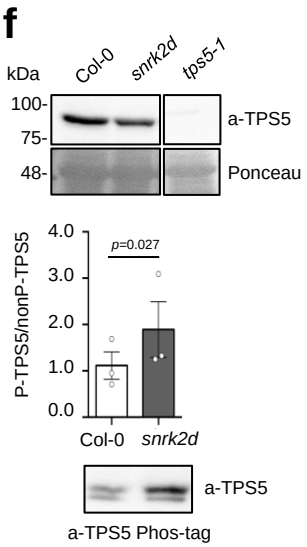
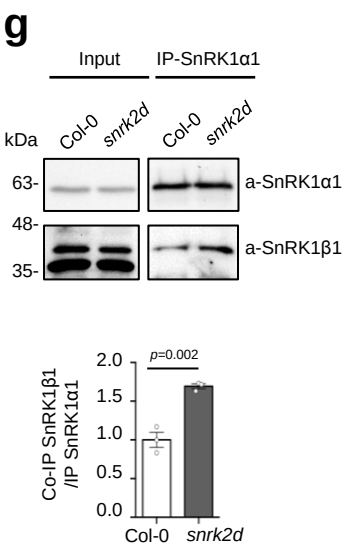
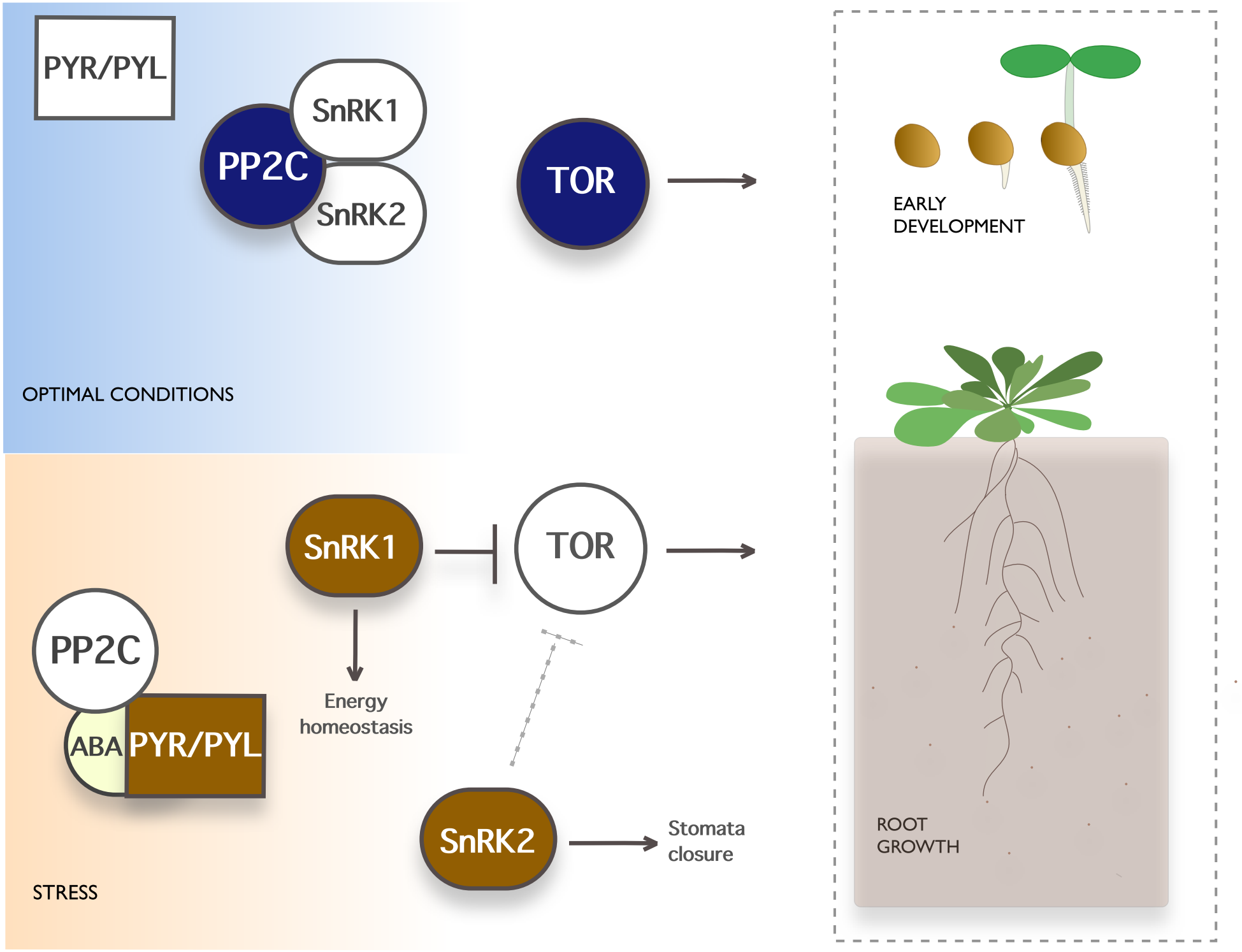
Mock
Radicle emergence (%)
Hours
Col-0
snrk2d
snrk2d/1α1
3 μM ABA
ABA insensitivity (%)
p=0.028
p=0.012
p=0.014
p=0.001
b
3 μM ABA
Greening (%) 16 days
p=0.019
c
Col-0
snrk2d
snrk2d/1α1
Mock
1 cm
5 μM ABA
PR length
LR density
MOCK
ABA
ABA insensitivity (%)
d
64%
81%
99%
mock
0.2 μM AZD
0.5 μM AZD
PR growth (% from mock)
0.2 μM
0.5 μM
[AZD8055]

Figure 3 (cont.)








PYR/PYL
SnRK1
PP2C
SnRK2
TOR
EARLY
DEVELOPMENT
OPTIMAL CONDITIONS
SnRK1
TOR
PP2C
ABA
PYR/PYL
Energy
homeostasis
SnRK2
Stomata
closure
ROOT
GROWTH
STRESS